Frequencies of Lipopolysaccharide Core Types among Clinical Isolates of *Escherichia coli* Defined with Monoclonal Antibodies

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Mouse monoclonal antibodies (MAbs) specific for the lipopolysaccharide (LPS) core types R1, R2, and R3 of *Escherichia coli* and a cross-reactive MAb that binds to the LPS core of almost all *E. coli* were used in ELISA to determine the frequency of cores resembling R1, R2, and R3 in strains of *E. coli* isolated from clinical samples (blood and urine specimens) and from the feces of asymptomatic individuals. Of the 180 wild-type isolates, 123 were assigned to R1 core type, 14 to R2, and 18 to R3. Twenty-five wild-type *E. coli* isolates could not be assigned to a particular core type and may have either an R4 or K12 core or a previously unrecognized core type. R1 core type was associated with O types 1, 4, 6, 8, and 18 and with K1 or K5 capsules. R3 was associated with O15. O75 isolates could be of either R1 or R2 core type.

The lipopolysaccharide (LPS) core region of *Escherichia coli* has a conserved overall structure, with an inner 2-keto-3-deoxy-octonate–heptose region and an outer hexose region. Most of the variation that does occur is in the outer part of the core, furthest from lipid A. In wild-type smooth *E. coli*, the core region can occur in at least four different forms, known as R1, R2, R3, and R4 [1–3]. The naturally rough strain K12 has a different core region [4].

The O antigen of smooth LPS may mask some of the epitopes and phage attachment sites in the core region. The chemical, serologic, and phage typing methods that have been used to define the different core types of *E. coli* are therefore not directly applicable to wild-type smooth strains. The frequency with which these different core types occur in wild-type *E. coli* is therefore not known.

Little is known about the association between core types and O serotypes of *E. coli*, except that the original R1 and R2 rough mutants were both derived from O8 strains [1] and that the original R3 mutant was derived from an O111 strain [2]. There is no published evidence on the relationship of LPS core types to capsular types or to virulence. Information about the distribution of O and K antigens has proved to be useful, for example in identifying and understanding enteropathogenic strains [5] and in suggesting novel therapeutic approaches to bacteremia [6]. Information about core types might be equally interesting.

We therefore set out to produce a panel of mouse monoclonal antibodies (MAbs) that would react with specific LPS core types of *E. coli* and other gram-negative bacilli with related core structures. We wished to use these MAbs as a means of determining the LPS core types of a collection of wild-type *E. coli*.

Materials and Methods

Bacteria and bacteriophages. *E. coli* R1 (F470), R2 (F576), R3 (F653), R4 (2513) and K12 (2131) and rough-specific phages FO, Br10, C21, 6SR, and T4 were obtained from G. Schmidt (Forschunginstitut, Borstel, Germany). *E. coli* O1, O2, O4, O6, O12, O15, O18, and O75 were obtained from A. S. Cross (Walter Reed Army Institute, Washington, DC). Phages specific for *E. coli* capsular types K1 (φK1GS) and K5 (φK5DG) were obtained from A. P. Roberts (Charing Cross and Westminster Medical School, London). Blood culture and urine isolates were obtained from the Clinical Bacteriology Laboratory, Department of Medical Microbiology, Edinburgh University, from routine clinical specimens. The blood culture isolates were all of the gram-negative organisms isolated in a 1-year period. Twenty-one fecal isolates of *E. coli* were obtained, each from a separate healthy volunteer. Rough mutants of *Salmonella minnesota* (R60), *Salmonella typhimurium* (1542), *Klebsiella pneumoniae* (M10B), and *Pseudomonas aeruginosa* (PAC608) and wild-type smooth *Shigella sonnei* (colicin types 4/2 and 2/1) and *Shigella flexneri* (serotypes 1a and 3) were freeze-dried stock cultures held in our laboratory.

MAbs. MAbs were produced by standard methods [7]. The details of immunization schedules and selection procedures are to be published elsewhere. All were reactive in ELISA with polymyxin-complexed rough LPS [8]. All MAb preparations were supernatants of hybridoma cell cultures grown in RPMI 1640 supplemented with 5% fetal calf serum. Antibody concentration was in the range of 10–50 μg/mL.

ELISA. Overnight nutrient broth cultures of bacteria were washed and resuspended in saline to give an OD of 0.5 at 525 nm and heated for 30 min at 100°C. Cells were then diluted

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1/20 in coating buffer (sodium carbonate-bicarbonate, pH 9.6, with 0.05% sodium azide) and 100 μL/well was added to Nunc (Roskilde, Denmark) Polysorb strips. After overnight incubation at room temperature, plates were washed three times with wash buffer (0.05% Tween 20 and 0.05% sodium azide in PBS, pH 7.4) and postcoated with 5% (wt/vol) bovine serum albumin (BSA) in coating buffer overnight at room temperature. Plates were then washed and stored at −20°C.

MAbs were diluted 1/250 in dilution buffer (4% [wt/vol] polyethylene glycol, 0.05% sodium azide, and 0.05% tween 20 in PBS, pH 7.4). Diluted MAb (100 μL) was added to wells and incubated for 90 min at 37°C. Plates were washed three times, and urease-conjugated anti-mouse immunoglobulin (Zymed, Cambridge, UK; diluted 1/500 in dilution buffer with 0.5% BSA, 100 μL/well) was added and incubated for a further 90 min. Plates were then washed three times in wash buffer and three times in distilled water; urease substrate solution (Seralab, Crawley Down, UK; 100 μL/well) was added and plates were incubated for 90 min at room temperature and read at 590 nm. An OD of >0.8 was considered positive.

PAGE and immunoblotting. LPS was prepared by proteinase K (protease type XI; Sigma, Poole, UK) digestion of lysed bacteria [9] and separated on 14% polyacrylamide gels [10] with SDS omitted from stacking and separating buffers. Gels were then silver stained [9] or blotted to nitrocellulose membrane (0.2-μm pore size) [11]. Blots were blocked with 3% (wt/vol) gelatin in TRIS-buffered saline, pH 7.5 (TBS), incubated for 3 h in a 1/10 dilution of MAb in 1% gelatin in TBS followed by a 1-h incubation with horseradish peroxidase anti-mouse IgG conjugate (ICN, High Wycombe, UK; diluted 1/1000), and developed with horseradish peroxidase color reagent (Bio-Rad, Hemel Hempstead, UK).

Serum sensitivity of bacteria. Serum from one donor was stored at −70°C. This serum had IgG antibodies to LPS core at a level equal to the 65th centile of the normal population [8]. Overnight broth cultures were washed and resuspended in complement fixation test (CFT) buffer (Oxoid, Basingstoke, UK) at room temperature to an OD of 0.5 at 525 nm. This suspension was then diluted 1/5000 in CFT buffer, and 10 μL was added to 70 μL of CFT buffer and 20 μL of freshly thawed serum or heated (56°C, 30 min) serum. After 1 h at 37°C, duplicate 20-μL samples were spread on the surface of nutrient agar plates, and colonies were counted after overnight incubation. If the number of colony-forming units in the fresh serum was <10% of the number in the heated serum, the organism was considered to be serum sensitive.

O typing. *E. coli* isolates from blood and urine cultures were screened for common O antigens, by ELISA with absorbed O typing sera (Difco, Detroit) to O1, O2, O4, O6, O7, O12, O15, O18, O22, and O75 (diluted 1/1000) and by tube agglutination at a final dilution of 1/80 with unabsorbed sera to O8 and O9 (Statens Seruminstitut, Copenhagen). O types were confirmed by conventional tube agglutination [12].

Phage typing. Nutrient agar plates were surface inoculated by flooding with overnight broth culture and allowed to dry. Phage suspension (10 μL) was dropped onto the agar and incubated overnight. A clear zone of lysis was scored as sensitive.

Table 1. Reactivity of selected monoclonal antibodies (MAbs) in ELISA.

	Reactivity with *E. coli* strain							
MAb	R1	R2	R3	R4	K12	O12	O15	O18
SZ 43 3.4.8	+	−	−	−	−	−	−	+
H4 361.23	−	+	−	−	−	+	−	−
W4 434.07	−	−	+	−	−	−	+	−
SZ 27 150.3	+	+	+	+	+	+	+	+

NOTE. +, strong reactivity (OD >0.8); −, no or weak reactivity (OD <0.15).

Statistics. All *P* values are two-tailed results calculated by Fisher's exact test.

Results

Selection of core-specific MAbs. A preselected panel of LPS-reactive MAbs was tested by ELISA against heat-killed *E. coli* rough strains R1, R2, R3, R4, and K12; *E. coli* smooth strains O1, O2, O4, O6, O12, O15, O18, and O75; and the rough mutants of *K. pneumoniae*, *P. aeruginosa*, *S. minnesota*, and *S. typhimurium*. MAbs that reacted with a restricted range of R types and also with some smooth strains of *E. coli* were thought likely to be useful in core typing wild-type smooth strains. Four such distinct patterns of reactivity were observed with more than one antibody. R1-specific MAbs (e.g., SZ 43 3.4.8) reacted with R1 and with *E. coli* O1, O2, O4, O6, O18, and O75; R2-specific MAbs (e.g., H4 361.23) reacted with R2 and O12; R3-specific MAbs (e.g., W4 434.07) reacted with R3 and O15; cross-reactive MAb SZ 27 150.3 reacted with all the *E. coli* rough and smooth strains and with the *Salmonella* rough mutants but not with the *Klebsiella* or *Pseudomonas* rough mutants (table 1).

Other patterns were observed, including a group of MAbs that reacted with R2 and K12, with R2 and the *Salmonella* rough mutants, or with all of these strains. However, no MAb reacted with K12 or R4 but not with other rough mutants. None of the MAbs used in this study reacted with the *Klebsiella* or *Pseudomonas* rough mutants tested.

The specificity of MAbs for LPS core was confirmed, for the MAbs given as examples above, by immunoblotting against LPS (proteinase K extracts) from *E. coli* R1, R2, R3, R4, K12, O12, O15, and O18. The O12, O15, and O18 strains were chosen as they seemed, from the ELISA data, to represent smooth strains with R2, R3, and R1 core types, respectively. As seen in figure 1, the immunoblots showed reactivity of each MAb with a band corresponding to the LPS core of each of the organisms with which it reacted in ELISA.

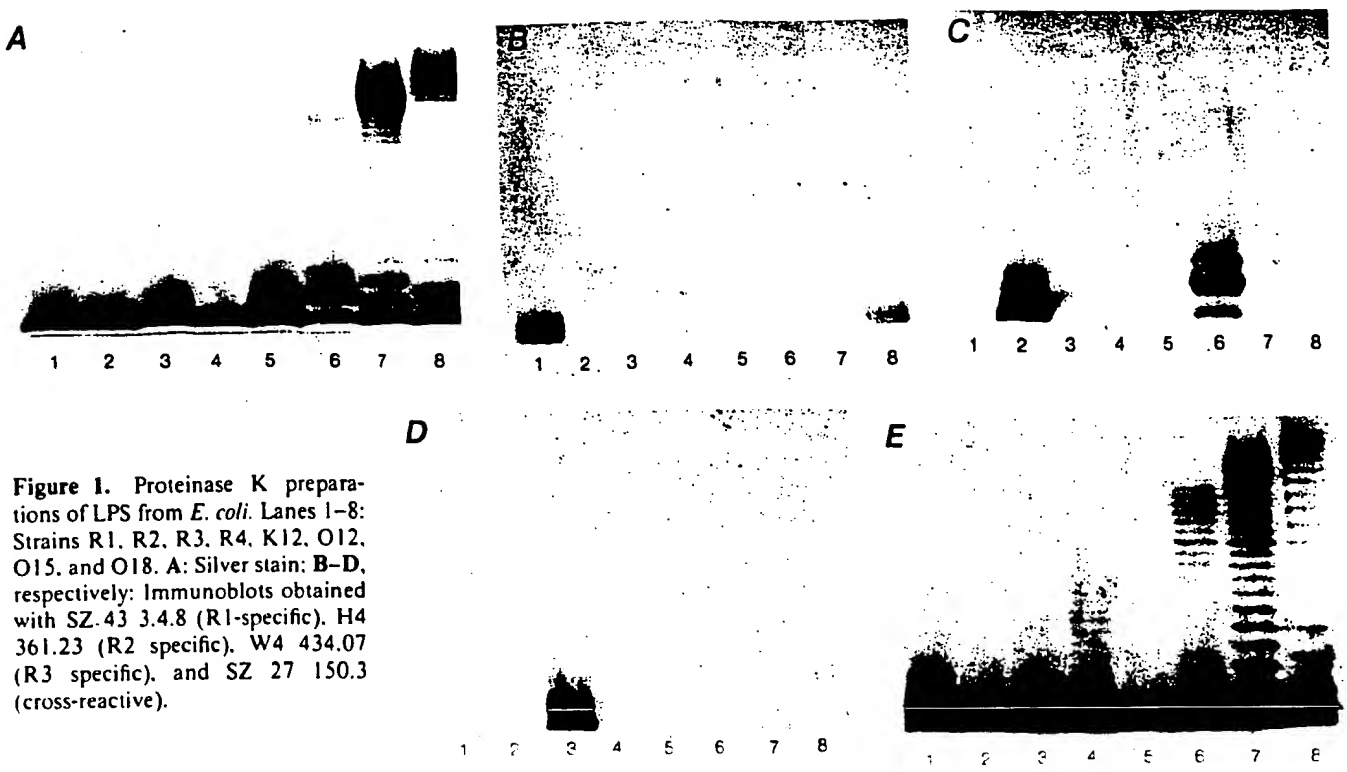

Figure 1. Proteinase K preparations of LPS from *E. coli*. Lanes 1–8: Strains R1, R2, R3, R4, K12, O12, O15, and O18. **A**: Silver stain; **B–D**, respectively: Immunoblots obtained with SZ 43 3.4.8 (R1-specific), H4 361.23 (R2 specific), W4 434.07 (R3 specific), and SZ 27 150.3 (cross-reactive).

With the rough mutants, a band was seen that corresponded to the single band seen in the silver stain. With the smooth strains, the R1 and R3 MAbs again reacted with a single band corresponding to the fastest-moving band seen in the silver stain. This band consists of LPS core that has not been substituted with O antigen [13]. The R1 antibody reacted in the same way in blots with the O1, O2, O4, O6, and O75 strains (not shown). The R2-specific MAb H4 361.23 and the cross-reactive MAb SZ 27 150.3 reacted with the unsubstituted core band and also with the "ladder" pattern of bands corresponding to O-substituted LPS core. MAb SZ 27 150.3 reacted weakly in the blot with bands in the R1 and R4 LPS preparations that were not visible in the silver stain. We believe these bands represent production, due to a leaky mutation, of small quantities of O-substituted LPS that are not detected by the silver stain but are detected by immunoblot.

Binding of anti-LPS core MAbs to gram-negative bacilli other than E. coli. Four *Shigella* strains were tested in ELISA, and all reacted with the cross-reactive MAb. In addition, both *S. sonnei* strains reacted with the R1-specific MAb, while the *S. flexneri* strains reacted with the R3-specific MAb. This is in agreement with previous studies of LPS core in these species [14–16]. Blood culture isolates of other gram-negative genera (*Acinetobacter*, 7; *Citrobacter*, 5; *Enterobacter*, 18; *Klebsiella*, 14; *Proteus*, 9; *Providencia*, 1; *Pseudomonas*, 8; *Salmonella*, 2; and *Serratia*, 5) were also tested in ELISA with the core type–specific and cross-reactive MAbs. The only positive reaction observed was between the two *Salmonella* isolates and the cross-reactive MAb. The cross-reactive MAb SZ 27 150.3 was tested in an immunoblot against LPS (proteinase K extracts) from single blood culture isolates of *K. pneumoniae*, *Proteus mirabilis*, *P. aeruginosa*, *Enterobacter cloacae*, and *Citrobacter freundii*. No reaction was observed with any of these isolates, confirming the lack of reactivity seen in ELISA.

Frequency of MAb-defined core types in wild-type isolates of E. coli. *E. coli* isolates from blood cultures (n = 79), urine cultures (n = 80), and fecal specimens from asymptomatic volunteers (n = 21) were tested in ELISA with core type–specific and cross-reactive MAbs. The cross-reactive MAb reacted with all of the isolates tested except for two of the urine isolates. When the strains reacting with the cross-reactive MAb were tested with the R1-, R2-, and R3-specific MAbs, each MAb reacted with a mutually exclusive group of isolates, which were classified as R1, R2, and R3, respectively. Figure 2 illustrates these results for the fecal isolates. Of the two urine isolates that did not react with the cross-reactive antibody, one reacted with the R1 antibody. These and the isolates that did not react with any of the core-specific antibodies are referred to as RNC (not classified). The numbers of wild-type isolates in the four categories are given in table 2. R1 was the most common classification overall (123/181, 68%) and was more common among isolates from

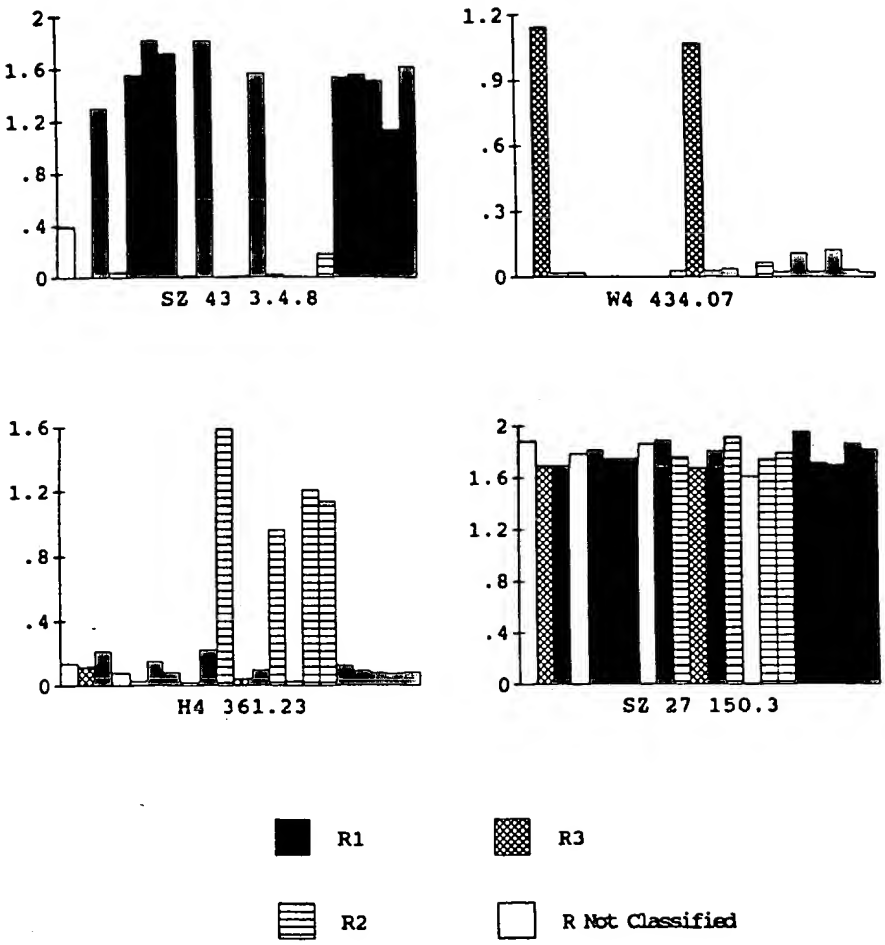

Figure 2. Reactivity (ELISA optical density) of antibodies with 21 fecal *E. coli* showing mutually exclusive reactions. Shading indicates core type to which each strain was assigned.

urine (62/80, 78%) than among those from blood culture (48/79, 61%, $P = .017$) or feces (11/21, 52%, $P = .030$).

Those MAbs that had reacted with R2, K12, and *Salmonella* rough mutants reacted with all of the R2 group of wild-type isolates but also reacted with variable numbers of the RNC isolates. This showed that the RNC group was heterogeneous but did not permit clear subdivision of this group.

Relationship between MAb-defined core type and sensitivity to rough-specific and K-specific phages (table 3). The collection of wild-type *E. coli* was tested for sensitivity to the rough-specific phages that have been used to distinguish between the different R types of *E. coli* [3], since it was thought that the pattern of reactivity might support the MAb-defined R typing scheme. Fifty of the 180 isolates tested were sensitive to one or more of the rough-specific phages. They were found among each of the R types defined with MAbs. They included isolates that were O typeable and produced a ladder

Table 2. Frequency of core types in wild-type isolates of *E. coli* determined by binding of core-type–specific monoclonal antibodies (MAbs) in ELISA.

Source	No. (%) of strains assigned to MAb-defined core type				Total
	R1	R2	R3	RNC	
Blood culture	48 (61)	6 (7)	12 (15)	13 (16)	79
Urine	64 (81)	4 (5)	4 (5)	8 (10)	80
Feces	11 (52)	4 (19)	2 (10)	4 (19)	21
Total	123 (68)	14 (8)	18 (10)	25 (14)	180

NOTE. NC, not classified.

Table 3. Sensitivity of 180 *E. coli* of different core types to rough-specific, K1-specific, and K5-specific phages.

Core type (*n*)	No. of strains sensitive to phage							
	F0	Br10	C21	6SR	T4	Any R+	K1	K5
R1 (123)	0	8	17	5	28	40	19	20
R2 (14)	0	2	0	1	3	3	0	1
R3 (18)	0	0	0	0	2	2	0	1
RNC (25)	0	3	2	2	3	5	3	2
Total (180)	0	13	19	8	36	50	22	24

NOTE. Any R+, any rough-specific phage; NC, not classified.

Table 4. O type of *E. coli* of different core types from blood and urine cultures.

Core type (*n*)	No. of each core type associated with O type										
	O1	O2	O4	O5	O6	O7	O8	O15	O18	O75	NT
R1 (112)	11	0	6	1	26	3	3	0	10	6	46
R2 (10)	0	0	0	0	0	0	0	0	0	3	7
R3 (16)	0	0	0	0	0	0	0	3	0	0	13
RNC (21)	1	2	0	0	0	2	0	0	0	0	16
Total (159)	12	2	6	1	26	5	3	3	10	9	82

NOTE. NT, not typed by available O-specific sera; NC, not classified.

pattern on silver stain of LPS, as was found by Cross et al. [6]. C21 sensitivity, a marker for the R1 rough mutant, was found in 19 isolates (17 R1 and 2 RNC). FO sensitivity is a marker for R2 rough mutants, but no isolate was FO sensitive. There was no other discernible relationship between R type and sensitivity to particular rough-specific phages.

Forty-six of the 180 isolates were sensitive to one of the two K-specific phages. The R1 isolates accounted for 19 of the 22 K1 isolates and 20 of the 24 K5 isolates found.

Relationship between MAb-defined core type and O type among the urine and blood culture isolates. The urine and blood culture isolates of *E. coli* were tested for 12 O types found commonly among *E. coli* from blood culture [6, 12] or urinary tract infection [17] (table 4). The overall proportion of isolates that belonged to one of these O types was much higher among those of R1 core type (66/112, 58%) than among the other core types (11/47, 23%, $P = .0005$). Some constant associations between O type and core type were apparent: all 26 O6 isolates were R1, and all 3 O15 isolates were R3. In contrast, O75 isolates were found among both the R1 and the R2 core types. The proportion of the R1 isolates that belonged to one of the common O types was greater among the blood culture isolates (32/48, 67%) than among the urine culture isolates (34/64, 53%). This difference is not statistically significant ($P = .177$), but the number of R1 isolates not belonging to the common O types could account for the greater proportion of R1 isolates among the isolates from urine.

Relationship between R type, serum resistance, and LPS chemotype among blood culture isolates. Serum resistance, which is a property of most invasive strains of *E. coli* [6], was determined for all the blood culture isolates; 21 (26%) of 79 were serum sensitive. There were serum-sensitive isolates of all the core types, but serum sensitivity was slightly less common among the R1 isolates (9/48, 19%) than among the other R types (10/31, 32%). This difference may have been due to the association of R1 core with K1 and K5 capsule, since all of the 11 K1 isolates and all but 1 of the 10 K5 isolates from blood cultures were serum resistant.

Seventy of the 79 blood culture isolates produced a typical ladder pattern of LPS in silver stains of proteinase K digests (data not shown). The other 9 showed only one or a few fast-moving bands, one corresponding to the unsubstituted core. Three isolates were R1, 3 were R2, 2 were R3, and 1 was RNC.

Discussion

We have described a group of MAbs that are selective for the LPS core of *E. coli*. Some of the MAbs also bind to *Salmonella* and *Shigella* strains that share *E. coli* core structures. The MAbs do not bind to a range of other gram-negative bacilli, confirming that the binding observed was specific. We have shown that the binding of these antibodies in ELISA to bacterial cells heated at 100°C corresponds to their binding to unsubstituted (and in some cases O-substituted) LPS core in immunoblots.

Unsubstituted LPS core is present in smooth bacterial cells but may not be readily accessible to MAbs in standard laboratory cultures [18, 19]. Core epitopes are, however, much more accessible on bacterial cells grown in serum or in a magnesium-depleted medium, which is thought to mimic the conditions in the infected host [20]. Core epitopes also become more accessible in bacterial preparations heated at 100°C [21, 22]. The expression and accessibility of core epitopes and the possible existence of cross-reactive epitopes have important implications for immunotherapy of gram-negative sepsis [23].

We have examined the binding of selected MAbs to heated bacterial cells in ELISA. We could recognize three mutually exclusive groups among *E. coli* isolates and labeled them R1, R2, and R3 after the corresponding rough mutants. A fourth group, which could not be assigned to a particular core type, was referred to as RNC and is probably of heterogeneous core type. We included in the RNC group the strain that reacted with the R1-specific antibody but not with the cross-reactive MAb. If this strain had been of R1 core type, a reaction with the cross-reactive MAb would have been expected.

We anticipate that each of the groups R1, R2, and R3 defined with MAbs probably has the core structure of the named rough strain, though this has not been confirmed by chemical analysis. The presence of C21 sensitivity (a marker for R1 rough mutants) among the wild-type isolates of MAb-defined R1 rather than R2 or R3 core type supports our view that the MAb-defined core types are the same as the reference rough strains. Two RNC isolates were also C21 sensitive, but the specificity of these phages for particular core types is not based on an independent knowledge of their receptor sites, and it is not known how they might react with as-yet-unidentified core types of *E. coli*. Phage typing is of little value in determining the core type of wild-type isolates since most are resistant to all of the phages used.

No isolates of R4 or K12 core type were positively identi-

fied. This may be explained by the lack of suitable specific MAbs. We did have MAbs reactive with K12, but these also reacted with R2 and did not bind to a clearly defined group of wild-type isolates other than those that reacted with the R2-specific MAbs. The RNC group may include isolates with R4 or K12 core but probably also contains one or more as-yet-unidentified core types.

Comparison of the structural formulas [15] with the specificities of our MAbs could suggest binding of the MAbs to particular sugar residues. R1 and R3 each have a unique terminal branching hexosyl unit (β1-3-linked glucose and α1-3-linked *N*-acetylglucosamine, respectively) that may be the epitope for the R1 and R3 antibodies. R2 and *Salmonella* core share a terminal α1-2-linked *N*-acetylglucosamine, while R2 and K12 have in common the next three sugar residues. These may therefore be the epitopes for the MAbs that bound to these combinations of strains. A unique binding site for the R2-specific antibody is not obvious from the structural formulas. From the calculated three-dimensional structure of the different core types [24], however, it appears that the branch-terminating α1-6 galactose present in R2, *Salmonella* core, and K12 may be in a different orientation in R2 and may therefore be the R2-specific epitope. It is of note that while most of the core-specific MAbs reacted in immunoblot only with unsubstituted core, the R2-specific MAb also reacted with O-substituted core, giving a ladder pattern in immunoblots. This implies that the R2-specific epitope is the only one not altered or masked by the attachment of the O polysaccharide. Further study of the binding site of these antibodies may therefore be useful in elucidating the three-dimensional structure of LPS core and in attempts to define the attachment site of the O polysaccharide.

The binding of the cross-reactive MAb SZ 27 150.3 to almost all of the *E. coli* isolates and to the *Salmonella* and *Shigella* strains, together with the pattern of binding of the core-specific MAbs, supports the view that these organisms share closely related LPS cores. The lack of binding of any of the antibodies to other genera suggests that these core structures are not distributed more widely. The structure of LPS core has not been determined for all the other genera, but it has been shown that the LPS cores of *Citrobacter* [25, 26] *Proteus* [27], and *Pseudomonas* [28] species are different from that of *E. coli*.

A MAb designated T6, which recognized the R2 core of *E. coli* and the core of *Salmonella* species, did not react with 25 clinical isolates of *E. coli* in Hong Kong [29], suggesting that the R2 core type was rare. We have found, however, that R2 accounted for 8% of our collection of *E. coli* isolates. This suggests that attempts to use MAb T6 in a capture ELISA [22] to detect *Salmonella* organisms in clinical samples may yield a significant number of false-positives because of the presence of *E. coli* of R2 core type.

The tendency for certain apparently distinct properties of *E. coli* to occur together [5] seems to apply to the relationship between core types and O and K types. It may be that there are chemical or structural factors limiting the ability of *E. coli* to express certain combinations of O and core types (e.g., O15 and R1) or it may be that some such combinations have a selective advantage in the host. The finding that there was greater proportion of R1 core type among the urine isolates and in particular that there was a greater proportion of R1 isolates not belonging to one of the common O types may indicate some relationship between R1 core type and unusual O types and pathogenicity in the urinary tract. Further studies are needed before firm conclusions can be drawn on this point.

Core typing with MAbs may be a useful addition to current typing methods for *E. coli*. The number of possible types is small, and the core type does not usually discriminate among strains of a given O and K type, but core typing may be useful in recognizing relatedness between groups of *E. coli*. We hope to use these antibodies to investigate the LPS core type of a wider range of *Shigella* and *E. coli* isolates from a variety of sources.

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